Colorectal Cancer Screening by Detection of Altered Human DNA in Stool: Feasibility of a Multitarget Assay Panel

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Background & Aims: **Assay of altered DNA exfoliated into stool represents an intriguing approach to screen for colorectal neoplasia, but multiple markers must be targeted because of genetic heterogeneity. We explored the feasibility of a stool assay panel of selected DNA alterations in discriminating subjects with colorectal neoplasia from those without.** ***Methods:*** **Freezer-archived stools were analyzed in blinded fashion from 22 patients with colorectal cancer, 11 with adenomas ≥1 cm, and 28 with endoscopically normal colons. After isolation of human DNA from stool by sequence-specific hybrid capture, assay targets included point mutations at any of 15 sites on K-*ras*, *p53*, and *APC* genes; Bat-26, a microsatellite instability marker; and highly amplifiable DNA.** ***Results:*** **Analyzable human DNA was recovered from all stools. Sensitivity was 91% (95% confidence interval, 71%–99%) for cancer and 82% (48%–98%) for adenomas ≥1 cm with a specificity of 93% (76%–99%). Excluding K-*ras* from the panel, sensitivities for cancer were unchanged but decreased slightly for adenomas to 73% (39%–94%), while specificity increased to 100% (88%–100%).** ***Conclusions:*** **Assay of altered DNA holds promise as a stool screening approach for colorectal neoplasia. Larger clinical investigations are indicated.**

Colorectal cancer remains the second leading cause of malignant mortality in industrialized nations, accounting for more than 10% of all cancer deaths.[1] Because of its orderly natural history and nonsurgical accessibility, colorectal neoplasia appears ideally suited for preventive interventions. However, screening efforts have had relatively little impact partly because of performance limitations and low compliance rates with current screening tools. More optimally tailored screening tools are needed that would exhibit the combined features of high sensitivity and specificity for early-stage cancers and large premalignant adenomas, broad acceptability by the

Stool testing merits further consideration because its theoretical potential has not yet been achieved. Stool tests are noninvasive, require no cathartic preparation, can be performed on mailed-in specimens without a mandated health center visit, and may reflect the full length of the colorectum. Fecal occult blood testing regularly performed over a decade or more may lower colorectal cancer mortality by 15%–33%, primarily by detecting cancers at an earlier stage.[2–4] However, many cancers and most premalignant adenomas do not bleed and are missed.[5,6] Furthermore, high false-positive rates as a result of frequent and trivial sources of occult bleeding lead to unnecessary colonoscopies, which drive up programmatic costs.[7–9] More sensitive and specific markers would improve the effectiveness and efficiency of stool screening.

Neoplasm-specific DNA alterations have been well-characterized[10,11] and represent intriguing candidate markers for stool screening. In contrast to blood, altered DNA arises from the neoplasm rather than from the circulation and is released into the lumen continuously via exfoliation rather than intermittently via bleeding.[12] Furthermore, DNA appears to be stable in stool[13] and amplification techniques permit detection of minute amounts of analyte. Several investigators have recovered mutant DNA in stools from patients with colorectal cancer or adenomas.[14–20] Assays used have typically analyzed mutations on a single gene, especially K-*ras* because of its small number of mutational sites. However, colorectal neoplasms are genetically heterogeneous[10,11]; no single mutation has been identified that is expressed across all colorectal neoplasms. Mutant K-*ras*, for example, is expressed by fewer than half.[10,11,21] Thus, multiple

Abbreviations used in this paper: L-DNA, "long" DNA; PCR, polymerase chain reaction.

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DNA alterations must be targeted to achieve high neoplasm detection rates.

An assay system was developed which targets a spectrum of DNA alterations that occur with colorectal neoplasia. This multicomponent assay panel targets point-mutations at any of 15 mutational hot spots on K-*ras*, *APC*, and *p53* genes; Bat-26, a marker of microsatellite instability[22,23]; and highly-amplifiable or "long" DNA (L-DNA). Observations by other investigators[17] of higher fecal DNA yields from patients with colorectal cancer than from controls provided the basis to include this latter marker in the assay panel.

The aim of this blinded clinical pilot investigation was to assess the potential of a multitarget fecal DNA assay panel to discriminate selected patients with colorectal neoplasia from those without neoplasia using colonoscopy as the criterion standard.

Materials and Methods

Design and Subjects

The investigation was approved by the Mayo Clinic Institutional Review Board and comprised 2 clinical pilot studies. Stools for each were selected from a freezer archive to yield subject groups with verified colorectal adenocarcinoma, colorectal adenomas ≥1.0 cm, and colonoscopically normal colons. Subjects were chosen to provide a balanced age and gender representation across groups and a mixed distribution of neoplasms from both proximal and distal colorectal sites (Table 1). Most patients with cancer had been referred with a known diagnosis or with a radiographically suspicious mass. All subjects with adenomas and normal colons were asymptomatic and undergoing surveillance because of either a family history of colorectal neoplasia or a previous personal history of neoplasia.

Pilot study 1 was conducted to explore the diagnostic discrimination of the fecal DNA assay panel by confirming the appropriateness of preset positivity levels for point mutations and BAT-26, by establishing a cutoff level of positivity for L-DNA, and by correlating specific mutations found in stool with those in matched tumors. In pilot study 2, the assay panel was applied using assay parameters established from pilot study 1, and the major focus of study 2 was to examine specificity in a separate group. As indicated in Table 1, 7 of the 10 patients with neoplasms in pilot study 2 had also been tested in pilot study 1. Because separate unthawed fecal aliquots were assayed on these 7 patients, within-stool reproducibility of assay results could be determined. All assays were performed by technicians blinded to the clinical data. On pathology review, 1 of the adenomas originally in pilot study 1 was found to have high-grade dysplasia with a focus of invasive cancer and was reassigned from the adenoma to the cancer group. In pilot study 2, 2 of the original 20 normal controls were excluded, one because of an inadequate colonoscopy because of the subsequent finding of a malignant ileal carcinoid tumor.

Table 1. Demographic and Colorectal Neoplasm Characteristics of Subject Groups

	Pilot 1	Pilot 2	Combined
Cancers			
n	21	8	22[b]
Sex (M/F)	11/10	4/4	11/11
Age (*yr*)[a]	69 (38–88)	73 (54–83)	70 (38–88)
Tumor site, prox/dist	10/11	4/4	14/15
Tumor size (*cm*)[a]	4.9 (2.6–11)	3.9 (2.5–11)	4.1 (2.5–11)
Tumor stage, Dukes AB/CD	13/8	5/3	13/9
Adenomas			
n	9	2	11
Sex (M/F)	4/5	1/1	5/6
Age (*yr*)[a]	69 (61–76)	74 (72–76)	73 (61–76)
Polyp site, prox/dist	5/4	1/1	6/5
Polyp size (*cm*)[a]	1.5 (1–5)	4 (1–7)	2 (1–7)
Normal colons			
n	10	18	28
Sex (M/F)	5/5	9/9	14/14
Age (*yr*)[a]	69 (53–77)	67 (50–74)	68 (50–77)

prox, proximal to splenic flexure; dist, splenic flexure or distal.
[a]Median (range).
[b]Because stools from 7 cancer patients from pilot 1 were repeated in pilot 2, total number of unique cancer patients was 22.

Stool Collection, Processing, and Storage

All stools had been collected within days before cathartic preparation for a scheduled colonoscopy, which served as the criterion standard. Any previous instrumentation had occurred ≥2 weeks before stool collections from colorectal cancer patients and ≥1 year for patients with adenomas and normal controls. To prevent toilet water artifact,[24] a plastic bucket device was used to collect whole stools. Stools in sealed buckets were received within 12 hours of defecation at the on-site processing laboratory where they were tested by Hemoccult (see below) and promptly frozen at −80°C in multiple aliquots. Frozen single fecal aliquots of at least 6 g per subject were sent in batches on dry ice for blinded DNA analyses at EXACT Laboratories (Maynard, MA).

Multitarget DNA Assay Panel

Total nucleic acid preparation. All stool samples were thawed at room temperature and homogenized in an excess volume (>1:10, wt, vol) of EXACT buffer A (EXACT Laboratories) using an EXACTOR stool shaker (EXACT Laboratories). After homogenization, a 4-g stool equivalent of each sample was centrifuged to remove all particulate matter, and the supernatants were incubated at 37°C after addition of proteinase K (0.5 μg/μL) and sodium dodecyl sulfate (0.5%). The supernatants were subsequently extracted with Tris-saturated phenol (GIBCO BRL, Grand Island, NY), phenol/chlo-

cleic acid was then precipitated (1/10 volume 3 mol/L NaAc and an equal-volume isopropanol), removed from solution by centrifugation, and resuspended in TE (0.01 mol/L Tris [pH 7.4] and 0.001 mol/L EDTA) buffer containing RNase A (2.5 μg/mL). For each group of samples prepared, process positive control samples as well as component negative controls were included.

Sequence-specific purification and amplification. Sequence-specific DNA fragments were purified from the total nucleic acid preparations by performing oligonucleotide-based hybrid captures. For each sample, 7 unique hybrid capture reactions were performed in duplicate. Each capture reaction was carried out by adding 300 μL of sample preparation to an equal volume of 6 mol/L guanidine isothiocyanate solution (GIBCO BRL) containing biotinylated sequence-specific oligonucleotides (20 pmol; Midland Certified Reagent Co., Midland, TX). After a 2 -hour incubation at 25°C, streptavidin-coated magnetic beads were added to the solution, and the tubes were incubated for an additional hour at room temperature. The bead/hybrid capture complexes were then washed 4 times with 1× B+W buffer (1 mol/L NaCl, 0.01 mol/L Tris-HCl [pH 7.2], 0.001 mol/L EDTA, and 0.1% Tween 20), and the sequence-specific captured DNA was eluted into 35 μL L-TE (1 mmol/L Tris [pH 7.4] and 0.1 mol/L EDTA) by heat denaturation.

Polymerase chain reaction (PCR) amplifications (50 μL) were performed on MJ Research Tetrad Cyclers (Watertown, MA) using 10 μL of captured DNA, 1× GeneAmp PCR buffer (PE Biosystems, Foster City, CA), 0.2 mmol/L dNTPs (Promega, Madison, WI), 0.5 μmol/L sequence-specific primers (Midland Certified Reagent Co., Midland, TX), and 5 U Amplitaq DNA polymerase (PE Applied Biosystems, Norwalk, CT). All sequence-specific amplification reactions were performed in identical thermocycler conditions. After an initial denaturation of 94°C for 5 minutes, PCR amplification was performed for 40 cycles consisting of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, with a final extension of 5 minutes at 72°C. For PCR product analysis, 8 μL of each amplification reaction was loaded and electrophoresed on a 4% ethidium bromide–stained NuSieve 3:1 agarose gel (FMC, Rockland, ME) and visualized with a Stratagene EagleEye II (Stratagene, La Jolla, CA) still image system (Figure 1).

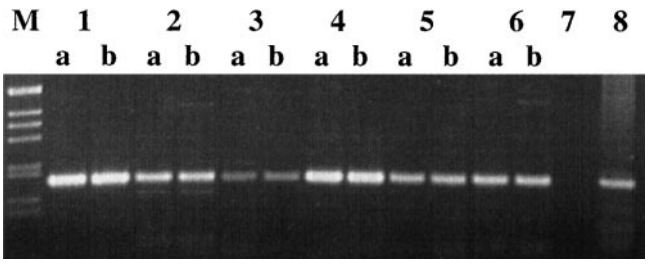

Figure 1. Agarose gel electrophoretic analysis of K-*ras* PCR products. Amplification results representing 6 unique stool DNA samples (1–6) amplified in duplicate (*lanes a + b*) with appropriate negative (*lane 7*) and positive (*lane 8*) control amplifications. Similar results were ob-

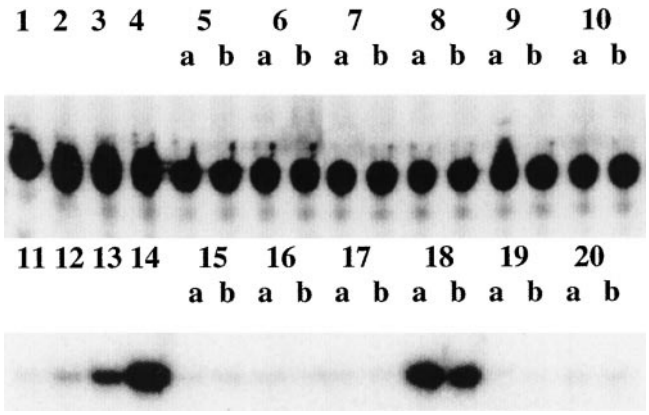

Figure 2. Point mutational results for *APC* codon 1378 position 1. Point mutation results representing 6 unique stool DNA samples (5–10 and 15–20) analyzed in duplicate (*lanes a + b*). Wild-type reactions (*lanes 1–10*) and corresponding mutant reactions (*lanes 11–20*) were analyzed by polyacrylamide gel electrophoresis. Positive wild-type results (*lanes 1–10*) served as internal sample specific controls. Each set of reactions (*lanes 11–20*) included mutation-specific positive controls representing 1% (*lane 13*) and 5% (*lane 14*) mutant DNA populations and negative control samples (*lanes 1, 2, 11,* and *12*) containing wild-type DNA only. Within this analysis, a single-stool DNA sample was positive for both wild-type (*lane 8, a + b*) and mutation-specific reactions (*lane 18, a + b*). All other stool DNA samples were positive for the wild-type reaction (*lanes 5–7, 9,* and *10, a + b*) and negative for the APC 1378 mutation (*lanes 15–17, 19,* and *20, a + b*).

Point mutation and Bat-26 analysis. The presence or absence of point mutations or Bat-26–associated mutations was determined by using a modified solid-phase minisequencing method.[25] Point mutation targets included codons K12p1, K12p2, and K13p2 on the K-*ras* gene; codons 1309 delta 5, 1367p1, 1378p1, and 1450p1 on the *APC* gene; and codons 175p2, 245p1, 245p2, 248p1, 248p2, 273p1, 273p2, and 282p1 on the *p53* gene. These targets were selected for assay because they correspond to the highest frequency mutational sites observed in available tissue databases. From these databases, theoretical diagnostic yields for cancer were estimated to be 44% for *p53*,[26] 41% for K-*ras*,[27] and 19% for *APC*[28] markers. For all gene targets, both wild-type and mutant-specific reactions were performed. Within the wild-type reactions, radionucleotide bases complementary to the wild-type base were added (Figure 2). For each point mutation–specific reaction, radionucleotide bases complementary to the expected mutant bases were added in addition to unlabeled dideoxy nucleotides complementary to the wild-type base (Figure 2). Bat-26 mutations associated with a deletion of 4–15 base pairs (bp) were identified by size discrimination of reaction products (Figure 3). We estimated that the theoretical yield by this microsatellite instability marker for cancer detection would be at least 15% based on reported observations in tissue.[29]

L-DNA analysis. L-DNA was performed by analyzing the relative intensity of each sample-specific PCR product.

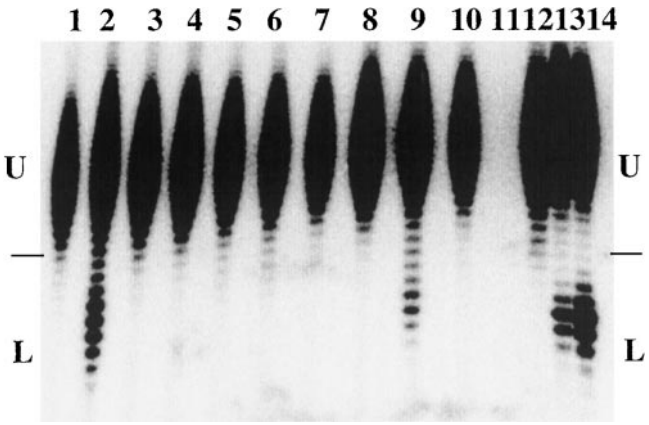

Figure 3. Polyacrylamide gel electrophoresis analysis for deletions within Bat-26. Results representing 10 unique stool DNA samples (*lanes 1–10*) analyzed for deletions within Bat-26. The upper (U) region of the gel contains reaction products representing the wild-type full-length product. Presence of reaction products within the lower (L) region of the gel is indicative of deletions within the Bat-26 polyA tract sequence. In addition to the stool samples analyzed (*lanes 1–10*), a no-DNA negative control (*lane 11*) and a wild-type-only DNA positive control (*lane 12*) were analyzed. Deletion-positive controls containing 1% (*lane 13*) and 5% (*lane 14*) mutant DNA (15 base deletions) were also analyzed within each assay to assure that resolution between wild-type and deleted sequences was achieved. Note that 2 of the samples (*lanes 2* and *9*) contain deletions within Bat-26, while all remaining samples contain the wild-type number of bases.

products were generated in duplicate (or 14 amplifications per subject) and independently scored by 2 technicians. PCR product intensities were scored as high (A), medium (B), or low (C) by visual examination of the gel image. Figure 1 illustrates examples of samples scored as A amplifications (lanes 1ab + 4ab), B amplifications (lanes 2ab, 5ab, and 6ab), and C amplifications (lane 3ab). The cutoff score to indicate a positive result was determined in pilot study 1.

Sequence information for all capture probes and primers will be available on request to the corresponding author.

Fecal Occult Blood Testing

All stools collected from subjects with adenomas and normal colonoscopy had been tested in blinded fashion by Hemoccult II (SmithKline Diagnostics, Sunnyvale, CA) immediately on receipt and before freezing. From a single stool, 2 aliquots sampled from opposite ends of the specimen were each smeared onto 2 windows of a Hemoccult II test card for a total of 4 windows. A single drop of peroxide catalyst was promptly added to each window, and a blue color reaction within 60 seconds on at least 1 of the 4 test windows was called a positive result for that stool. Positive and negative controls were tested with each run. Stools were selected from the archive for study without knowledge of Hemoccult status.

Tissue Processing and Assay

DNA from colorectal cancers in pilot study 1 was obtained at the Mayo Clinic after microdissection of sections blocks using a previously described extraction technique.[30] This DNA was sent to EXACT Laboratories where point mutation assays by the single-base extension method (see above) were performed in a blinded fashion.

Statistical Analysis

Sensitivity and specificity were estimated relative to the results of colonoscopy in the usual manner; 95% confidence intervals (CIs) for these estimated parameters were based on the exact binomial distribution. Comparisons of proportions between various subgroups were based on the Fisher exact test, and McNemar's matched-pairs test for proportions was used to compare sensitivities between the fecal DNA assay panel (with and without K-*ras*) and Hemoccult. Interobserver variability for L-DNA was assessed in pilot study 1 using a weighted κ statistic because the individual scores were ordinal in nature. The weighted κ statistic was estimated for each of the 7 PCR amplification products separately and for the pooled observations across all PCR amplifications. Among the 560 PCR amplifications scored, there were 7 instances in which 1 of the duplicate amplifications was noninformative; such instances were considered discordant so as to obtain a conservative estimate of interobserver variability.

Results

Analyzable human DNA was recovered in all subjects. When detected, mutant DNA accounted for 1%–24% of total human DNA recovered in stools from cancer patients and for 1%–7% from those with large adenomas.

Pilot Study 1

Cancers. At least 1 point mutation among the 15 targeted sites on K-*ras*, *APC*, and *p53* genes was present in stools from 11 (52%) of the 21 cancers. Bat-26 was positive in 4 cases, 1 of which also had a point mutation, yielding a detection rate of 14/21 (67%) when combined with point mutation components. Samples that were assigned an "A" score on >8 amplifications were considered L-DNA positive, because all of the colonoscopically normal controls fell below this cutoff. Using this definition, L-DNA alone was positive in stools from 14 (67%) of the 21 cancers. With all component markers together, the fecal DNA assay panel detected 19 (90%) of the 21 cancers (Table 2).

Tissue was available for DNA extraction and point mutation analyses on 19 of the 21 cancers. Point mutation results on tissue and stool were concordant in 12 cases (63 %): at least one pair of identical mutations was found in stools and matched tumors in 7 cases, and all targeted mutations were absent in both stool and tumors

Table 2. Positivity Rates of the Fecal DNA Assay Panel by Subject Group From Each Pilot Study and From Combined Studies: Component Markers and All Markers Together

						All markers	
	K-*ras*	*APC*	*p53*	Bat-26	L-DNA[a]	n (%)	95% CI
Pilot 1							
Cancers (21)	4	5	3	4	14	19 (90)	70%–99%
Adenomas (9)	0	3	0	0	5	7 (78)	40%–97%
Normals (10)	0	0	0	0	0	0 (0)	0%–31%
Pilot 2							
Cancers (8)	2	2	1	2	4	8 (100)	63%–100%
Adenomas (2)	1	0	0	0	1	2 (100)	16%–100%
Normals (18)	2	0	0	0	0	2 (11)	1%–35%
Combined studies							
Cancers (22)[b]	4	5	3	5	14	20 (91)	71%–99%
Adenomas (11)	1	3	0	0	6	9 (82)	48%–98%
Normals (28)	2	0	0	0	0	2 (7)	1%–24%
Combined studies (excluding K-*ras*)							
Cancers (22)[b]	—	5	3	5	14	20 (91)	71%–99%
Adenomas (11)	—	3	0	0	6	8 (73)	39%–94%
Normals (28)	—	0	0	0	0	0 (0)	0%–12%

[a]L-DNA refers to "long" or nonapoptotic DNA.
[b]Because stools from 7 cancer patients from pilot 1 were repeated in pilot 2, total number of unique cancer patients was 22.

detected in the stool only in 4 cases and the tissue only in 3 cases.

Adenomas and normal controls. The fecal DNA panel detected 7 (78%) of the 9 adenomas ≥1 cm. All positives were the result of *APC* mutations or elevated L-DNA (Table 2). The assay panel was negative in all 10 colonoscopically normal patients.

Interobserver variability for L-DNA. Perfect agreement between the 2 technicians scoring L-DNA ranged from 88% to 96% for duplicate testing on the 7 PCR amplification products; perfect agreement was >93% for 6 of the 7 products. Weighted κ values for individual PCR products ranged from 0.36 ± 0.09 to 0.74 ± 0.07; the pooled weighted κ value across all 7 PCR products was 0.58 ± 0.12.

Pilot Study 2

All 8 of the cancers and both of the adenomas were detected by the fecal DNA assay panel using the same assay parameters as in pilot study 1 (Table 2). Of the 7 cancer patients in this series who had also been evaluated by separate fecal aliquots in pilot study 1, at least 1 identical DNA alteration was reproduced in all 7 instances; all component markers of the assay panel were concordant in 5 instances and at least 1 marker was discordant in the other 2 instances. The assay panel was positive in 2 (11%) of the 18 colonoscopically normal controls. Both of these false-positive cases were a result of

Both Studies Combined

Sensitivity and specificity. Using the full panel of component markers, the sensitivity of the fecal DNA assay panel for the 22 cancers was 91% (95% CI, 71%–99%) and for the 11 adenomas ≥1 cm was 82% (95% CI, 48%–98%) with a specificity of 93% (95% CI, 76%–99%). If K-*ras* markers were excluded from the panel, then sensitivity for cancer was unaffected at 91% (95% CI, 71%–99%) but decreased slightly for adenomas to 73% (95% CI, 39%–94%) while specificity increased to 100% (95% CI, 88%–100%).

For all neoplasms (cancers and adenomas), L-DNA proved to be the most informative marker and alone detected 20 (61%) of the 33 unique lesions. Bat-26 and p53 markers were positive with cancer but not with adenomas in this initial series. In stools from the 20 cancer patients with a positive DNA assay panel, 3 component markers were positive in 3 cases, 2 markers positive in 5 cases, and a single marker positive in 12 cases. In stools from the 9 test-positive adenoma patients, 2 component markers were positive in 2 cases and a single marker was positive in the other 7.

In this highly selected subject group, the positive predictive value for colorectal neoplasia by the fecal DNA panel (excluding K-*ras* markers) was 100% (28/28) and the negative predictive value was 85% (28/33).

Clinical correlates. Positive results by the component marker Bat-26 were significantly associated with proximal colorectal cancer site, and an association of

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